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Syndrome of Lipoatrophic Diabetes, Vitamin D Resistant Rickets, and Persistent Müllerian Ducts in a Turkish Boy Born to Consanguineous Parents

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Congenital lipodystrophy (MIM 269700), persistent Müllerian ducts (MIM 261550), and vitamin D resistant rickets (MIM 277440) were observed in an 8½-year-old boy born to consanguineous parents. Measurements of hormone sensitive lipase activity from a sample of the suprapubic fat depot were normal. Although the insulin receptor appeared normal (including autophosphorylation), insulin action, assessed by induction of total mRNA, was decreased. The vitamin D receptor was normal in size and amount, with a slight decrease in affinity for $1,25(OH)_2D_3$. Induction of 24-hydroxylase, used as a measure of responsiveness to $1,25(OH)_2D_3$, was only mildly defective. Assessment of anti-Müllerian hormone (AMH) failed to show any abnormalities explaining the persistent Müllerian ducts. We speculate that a defect in general hormone action common to $1,25(OH)_2D_3$, insulin, and AMH may exist in this patient although we can not exclude the unlikely possibility that he is homozygous for two or three individually rare mutations. © 1996 Wiley-Liss, Inc.

KEY WORDS: lipodystrophy, vitamin D, rickets, persistent Müllerian ducts, male pseudohermaphroditism

INTRODUCTION

We present here the unusual occurrence of three rare hereditary disorders in a boy born to consanguineous parents. Congenital total lipodystrophy, or Berardinelli-Seip syndrome, is a rare autosomal recessive disease combining facial anomalies, virtual absence of subcutaneous fat, severe hyperlipidemia, insulin resistance and acanthosis nigricans [Berardinelli, 1954; Seip, 1959]. Hereditary vitamin D resistant rickets (HVDRR) is a rare autosomal recessive form of severe neonatal or infantile onset rickets linked to absence or dysfunction of vitamin D receptors or post receptor defects in hormone action [Hughes et al., 1991]. Persistent Müllerian duct syndrome (PMDS) is a rare autosomal recessive form of male pseudohermaphroditism with lack of regression of Müllerian derivatives leading to persistence of uterus and fallopian tubes in the presence of normal male genitalia [Guerrier et al., 1989]. The presence of three rare genetic diseases, all manifested by hormone resistance, raises a number of interesting pathogenetic and nosologic questions.

CLINICAL REPORT

This boy was the first child of Turkish healthy first cousins aged 21 and 19 years, who subsequently had a normal boy and a normal girl. No other relatives have insulin resistance, persistent Müllerian ducts, or mental deficiency. Birth occurred at term. Birthweight was 3 kg and there was no alopecia. After a few hours, the infant was found to be hypotonic with tremors. Hypocalcemia occurred. At 10 days, surgical exploration of a right inguinal hernia showed a uterus and

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Since the paper was submitted, a point mutation has been identified in the steroid binding domain of the VDR and a manuscript describing this work is in preparation.

Fig. 1. **a:** Early-onset severe rickets at 18 months (left). Note metaphyseal cupping. **b:** Disappearance of active rickets on follow-up X-rays at 5 years. Skeletal alterations of knee with gross enlargement, hypomineralization, and flaring (bottom). **c:** Surgical findings of uterus and fallopian tubes during orchidopexy.

fallopian tubes in the hernial sac. At 3 months, in association with poor sucking and vomiting, the infant developed muscular hypertonicity. Roentgenographic signs of rickets were present. Serum glucose was 118 mg/dl. A diagnosis of rickets was made and treatment with vitamin D_3 was started (4,800 IU/d) which failed to correct hypocalcemia and roentgenographic signs of rickets after 1 month. A trial with Rocaltrol® [1,25$(OH)_2$ Vitamin D_3] failed. At 7 months the boy was admitted to the hospital for failure to thrive, rickets, hepatomegaly, and hyperlipoproteinemia. Liver biopsy indicated massive steatosis.

Serum cholesterol concentration was 101 mg/dl, triglycerides 2,900 mg/dl (N < 150), and non-esterified fatty-acids (NEFA) 2.75 mEq/l (N, 0.13–0.44). Hyperchylomicronemia was noted on lipidogram. After discontinuing vitamin D treatment for 3 weeks, serum concentration of 1.25$(OH)_2D_3$ was 65 pg/ml (N, 20–80). Subsequently, extraordinarily high doses of 1.25$(OH)_2$ D_3 (Rocaltrol®, 30 μg/day) resulted in normalization of serum calcium to 9.5 mg/dl, with correspondingly very elevated serum concentrations of 1.25$(OH)_2D_3$ (1,700→ 2,600 pg/ml) (N, 20–80). A low fat diet was started, resulting in lowering of lipidemia (cholesterol, 194 mg/dl; triglycerides, 749 mg/dl; NEFA, 0.60; and total lipids, 827 mg/dl). Apolipoproteins A1 and B were 77 mg and 95 mg/dl, respectively. At 2 years, liver fibrosis and steatosis were found increased on a repeat biopsy. He underwent orchidopexy at 3 years. The uterus was removed and the gonads (Fig. 1) were found to be testes on histology. Male external genitalia were otherwise normal as well as bladder and vas deferens. The clinical data were consistent with the diagnosis of persistent Müllerian duct syndrome (PMDS). Catch-up growth was observed. Signs of rickets were no longer observed on skeletal roentgenograms. At 5 years, his weight was at +2 SD, height at +1 SD and head circumference 53 cm (80th centile). Treatment with Rocaltrol® was stopped with rapid (1 month) reappearance of clinical and radiological signs of rickets, thus prompting continuation of treatment (Fig. 1).

At 9 years, a pseudo-athletic build with apparent absence of subcutaneous fat, acanthosis nigricans, and

Fig. 2. General view of the patient at 9 years (left). Note prematurely aged habitus, pseudo-athletic build, acanthosis nigricans, and genu valgum. Facial appearance: hypertrichosis, prominent supraorbital ridges, and large mouth, with empty cheeks are acromegaloïd characteristics of lipoatrophic diabetes (upper right). Acanthosis nigricans and pachydermia in the axillae (middle right). Acrogeria (lower right).

peculiar facial appearance, including hypertrichosis, prominent supraorbital ridges, empty cheeks, and full lips (Fig. 2), suggested the diagnosis of total lipodystrophy. This was confirmed by skin biopsy indicating paucity of subcutaneous fat with enlarged empty adipocytes. At 10 years, serum insulin level was 637 μU/ml (N, 5–30). However, serum glucose concentration was 110 mg/dl and oral glucose tolerance test (50 g) resulted in persistent hyperglycemia (214 mg/dl at 30 minutes, 278 at 60 minutes, and 347 at 120 minutes), in contrast to a normal oral glucose tolerance test performed 1 year before. Growth hormone, somatomedin, gonadotropins, and dynamic LHRH test were normal. Apo A1, A2, and CII were at the lower limit of normal range. The serum concentration of anti-Müllerian hormone (AMH), using antibodies specific for human AMH [Carré-Eusebe et al., 1992] was 11 ng/ml, at the lower limit of normal values in this age group. Plasma lipoprotein lipase concentration after 100 U/kg I.V. heparin was within the normal range. The boy is presently 12 years old, attending a school for the mentally handicapped (IQ, 35–50), with fairly normal functioning within his family. He has a minor motor disability due to marked genua valga.

LABORATORIES STUDIES

Vitamin D receptors (VDR) were analyzed in cultured dermal fibroblasts as described by Malloy [1989, 1990]. In brief, fibroblasts were harvested and sonicated in KTEDM buffer (0.3 M KCL, 0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.005 M dithiothreitol, and 0.01 M sodium molybdate) on ice and cell extracts prepared by ultracentrifugation at 200,000g for 30 min at 4°C. Extracts were incubated with [^{3}H]1.25(OH)$_2$D$_3$ (Amersham, Arlington Heights, IL) in the presence or absence of a 250-fold excess of unlabeled hormone. Bound and free steroid were separated by hydroxylapatite. Protein was measured by the method of Bradford [1976]. Scatchard plots were performed according to previously reported methods [Scatchard, 1949]. Total cellular RNA was prepared from fibroblasts using 4 M LiCl, 8 M urea as previously described [Krishnan et al., 1991]. Total RNA samples of 10 μg were fractionated by electrophoresis on 1% agarose/0.66 M formaldehyde gels. Fractionated RNA was blotted to nylon (Hybond-N, Amersham, Arlington Heights, IL) by capillary action. The blots were hybridized with a random primed ^{32}P-labeled 1.3 kb *Accl-Kpnl* fragment of the rat 25-hydroxyvitamin D-24 hydroxylase cDNA probe [Ohyama et al., 1991]. To control for sample loading and transfer, the blot was similarly probed with a 0.9 kb *Eco* RI fragment of the ribosomal gene L7 cDNA [Seshedri and Campisi, 1990].

Isolation of partially purified insulin receptors from cultured fibroblasts and insulin stimulated receptor autophosphorylation was performed as previously described [van der Vorm ER et al., 1992]. Assessment of mRNA synthesis in response to insulin binding was performed according to already described methods [Frorath, 1985].

Hormone-sensitive lipase (HSL) activity was measured in crude fat-depleted homogenates (110,000g) of freshly frozen adipose tissue obtained from the suprapubic adipose tissue depot. The diacyglycerol analogue, 1(3)-mono-[^{3}H]oleoyl-2-0-oleyl glycerol, that is not metabolized by the monoacylglycerol lipase was used as the substrate [Fredrikson, 1981; Holm et al., 1989; Frayn et al., 1993]. The degree of activation of HSL by cyclic AMP-dependent protein kinase was estimated by incubation with the catalytic subunit of cyclic AMP-dependent protein kinase, followed by assay with trioleoyl glycerol [Stralfors and Belfrage, 1983].

For analysis of the AMH gene, genomic DNA was prepared from EB virus-transformed lymphocytes, digested by either *Taq I* or *Hind III* restriction endonuclease and electrophoresed on a 1% agarose gel. After Southern transfer, the blot was probed with the AMH gene, which was amplified from genomic DNA by PCR using oligonucleotide primers designed to amplify the five exons and the exon-intron boundaries of the gene. PCR products were ligated into Sma I-digested phosphatase-treated M13 vector and sequenced by the dideoxynucleotide chain-termination method using [α-^{35}S] dATP and T7 DNA polymerase (Sequenase, USB), as previously described [Knebelmann et al., 1991].

RESULTS

Vitamin D Receptor (VDR)

Sucrose gradient analysis showed the patient's VDR to be of normal size, approximately 4S. The amount of receptors expressed in the patient's fibroblasts (60 fmol/mg protein) was in the normal range (30–70 fmol/mg protein). The binding affinity for [^{3}H]1.25(OH)$_2$D$_3$ was slightly decreased compared to normal (pt. 120 pM, N, 20–60 pM) (Fig. 3).

Cultured fibroblasts of the patient and control were treated with graded concentrations of 1.25(OH)$_2$D$_3$ and induction of 24-hydroxylase mRNA was measured as a marker of hormone responsiveness. The normal cells showed substantial 24-hydroxylase mRNA induction at 1 nM 1.25(OH)$_2$D$_3$ (Fig. 4). The patient's cells failed to show detectable 24-hydroxylase mRNA induction at 1 nM but exhibited marked stimulation at 10 nM of 1.25(OH)$_2$D$_3$ (Fig. 5).

Insulin

Specific insulin binding to cultured fibroblasts was increased and autophosphorylation of partially purified insulin receptors after insulin stimulation was within the normal range. It is seen in Figure 6 that receptors for insulin and IGF1 from the patient undergo a similar degree of hormone-induced autophosphorylation as receptors from control fibroblasts. These observations indicate normal insulin receptor properties. Total mRNA synthesis in response to insulin was clearly below the normal age-adjusted range (Fig. 7).

HSL

The HSL activity of suprapubic adipose tissue, expressed as fatty acid released/min/mg total protein at 37°C, was comparable in patient and control (139 and 98, respectively) and within the range normally found for human adipose tissue biopsies [Frayn et al., 1993]. The HSL of the adipose tissue homogenates was analyzed with regard to activation by cyclic AMP-dependent protein kinase. This in vitro activation was found

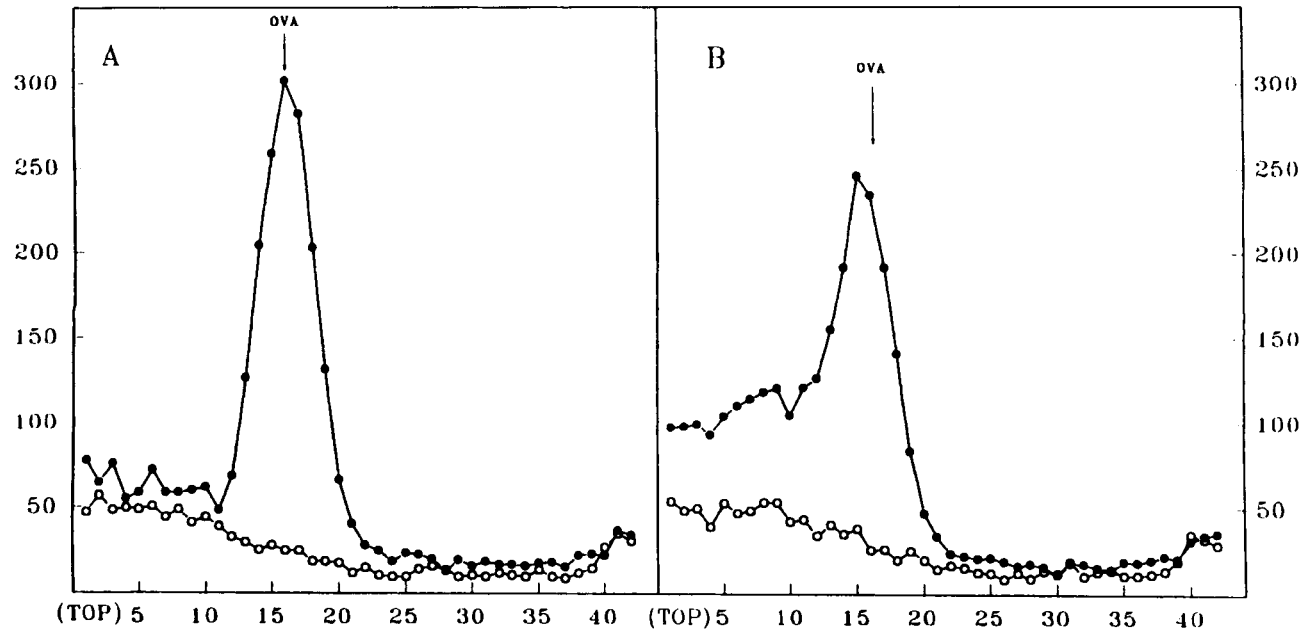

Fig. 3. Sucrose gradient centrifugation analysis of VDR: The VDR, extracted from cultured fibroblasts, was incubated with [^{3}H]1.25$(OH)_2D_3$ and analyzed on 6–20% sucrose gradients. [^{14}C] Ovalbumin (OVA) was used as standard (3.7S). (●): [^{3}H]1.25$(OH)_2D_3$ represents total bindings. (○): [^{3}H]1.25$(OH)_2D_3$ + 100-fold excess 1.25$(OH)_2D_3$ represents non-specific binding. Radioactivity (cpm) is expressed as a function of fraction number. **A:** Normal cells. **B:** Patient's cells.

to be 72% (72 ± 30 M ±SD, n = 3) for the control tissue, 68% (68 ± 28) for a partial purified rat adipose tissue HSL, used as reference material, whereas it was 110% (110 ± 64) for the lipodystrophic patient tissue.

AMH

After transfer of digested genomic DNA to a nylon membrane, hybridization with a probe spanning the full AMH gene failed to reveal any differences with con-

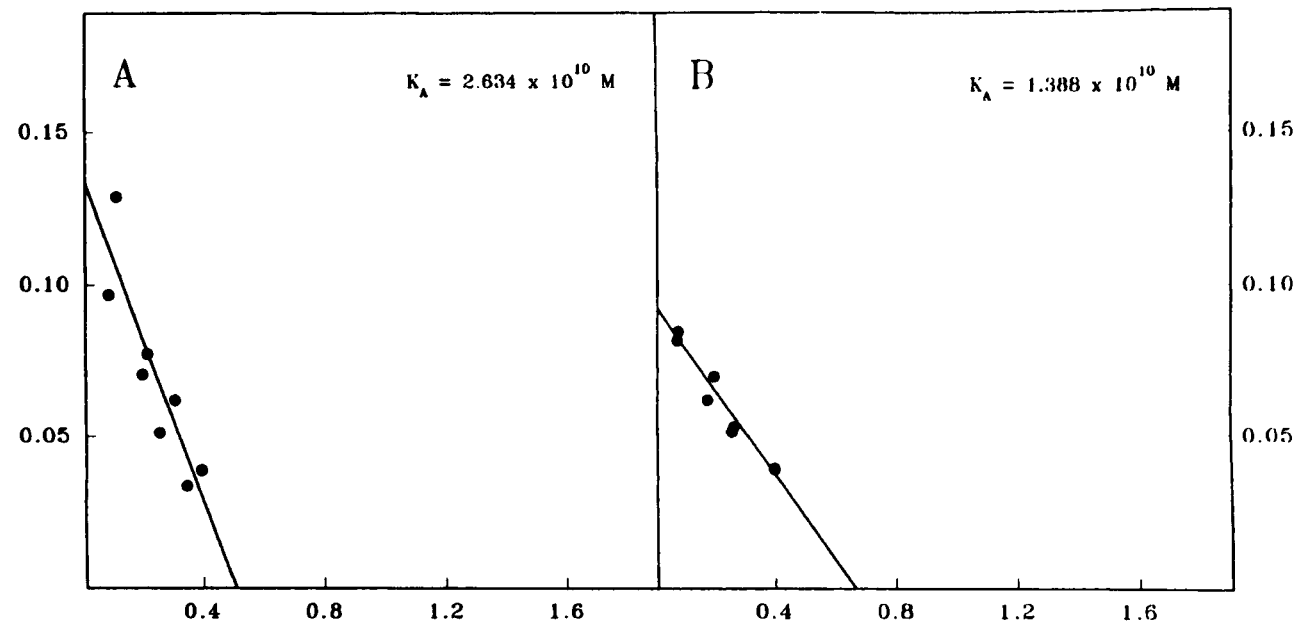

Fig. 4. Scatchard analysis shows normal abundance of VDR with slightly decreased affinity: VDR from cultured fibroblasts was incubated with graded concentration of [^{3}H]1.25$(OH)_2D_3$ and specific binding analyzed by Scatchard plot. The affinity (K_d) in the patient (**B**) was 120 pM which is somewhat lower than normal 20–60 pM (**A**). The abundance of VDR was 60 fmol/mg protein in the patient which is within the normal range (30–70 fmol/mg protein).

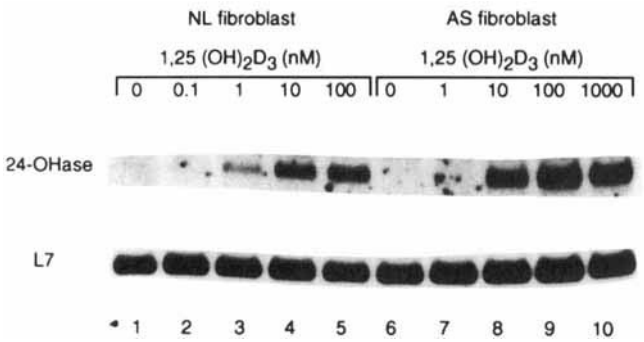

Fig. 5. Northern blot analysis of 25-hydroxy-vitamin D-24 hydroxylase induction: dermal fibroblasts from the patient (AS) and a normal control (NL) were treated for 6 hours with vehicle or with increasing concentration of $1.25(OH)_2D_3$. Total RNA was fractionated on 1% agarose formaldehyde gels, transferred to nylon, and probed with [^{32}P]-labeled rat 24-hydroxylase cDNA fragment. The blot was subsequently probed for the L-7 ribosomal protein as a control for RNA loading and transfer. **Lanes 1–5**, control cells; **lanes 6–10**, patient cells.

trol DNA concerning the size of the restriction fragments. Sequencing of the exons and intron-exon boundaries did not detect any abnormality.

DISCUSSION

Three different and apparently independent autosomal recessive disorders are simultaneously observed in this boy: Berardinelli-Seip syndrome, hereditary vitamin D resistant rickets (HDRR), and persistent Müllerian duct syndrome (PMDS). The concurrence of two autosomal recessive disorders has been described in some instances in highly inbred pedigrees but three autosomal recessive disorders in the same individual has not, to our knowledge, been reported so far.

First, concerning the syndrome of insulin resistance and lipodystrophy, we were unable to study it at the molecular level since its pathophysiology is currently poorly understood. However, some specific areas of insulin and lipid metabolism have been investigated in the present case. The patient exhibits diabetes, insulin resistance, and paucity of adipose tissue. In this form of lipoatrophic diabetes, which belongs to the largest group of insulin resistance syndromes, insulin binding to receptors has been studied in vitro on several occasions and reported to be normal or not significantly altered [Howard, 1981; Wachslicht-Rodbard et al., 1981; Whittaker et al., 1985; Kriauciunas et al., 1988; Magré et al., 1988]. Also the sequence of the insulin receptor gene is unchanged in these patients [van der Vorm et al., 1993]. By contrast, in leprechaunism, which appears clinically to be a more severe form of total lipodystrophy, a decreased binding of insulin to fibroblasts or an impaired stimulation by insulin of receptor autophosphorylation is frequently observed [Rechler, 1981]. Mutations in the insulin receptor gene have been reported in leprechaunism, leading in some cases to complete absence of normal receptors [Krook et al., 1993]. In fibroblasts from the present patient, insulin binding was increased compared to normal control values. Insulin activity, as assessed by total mRNA stimulation, was decreased. By contrast, autophosphorylation was normal. This points to a post-insulin binding defect distal to the autophosphorylation process. Other possi-

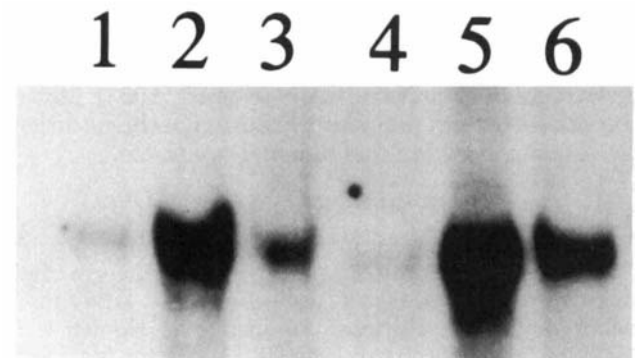

Fig. 6. Activity of receptors for insulin and IGF1 as reflected by hormone induced autophosphorylation. Partially purified receptors for insulin and IGF-1 from control fibroblasts [lanes 1–3 and patient's fibroblasts (lanes 4–6) were incubated without (lanes 1 and 4) or with 1 μM insulin (lanes 2 and 5) or 1 μM IGF-1 (lanes 3 and 6) in the presence of [^{32}P]-labeled ATP]. Receptors for insulin or IGF-1 were precipitated by specific antibodies and the immune precipitate was analyzed by autoradiography.

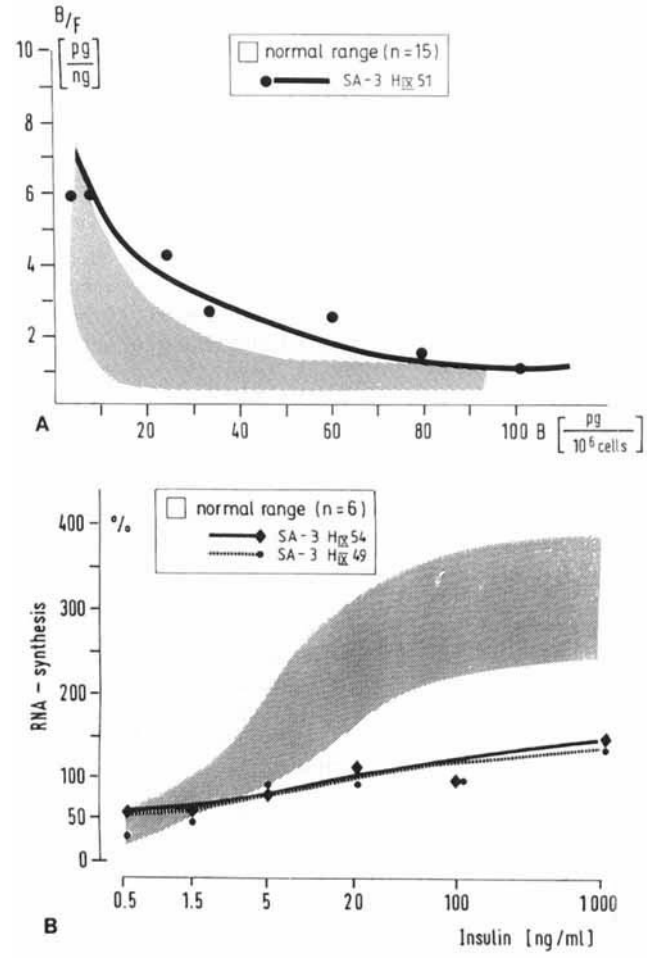

Fig. 7. **A:** Specific insulin binding on cultured fibroblasts plotted according to Scatchard. **B:** Insulin induced RNA synthesis in cultured fibroblasts of the index patient (●, Δ: two independent experiments) and six healthy controls.

ble explanations for the lack of antilipolytic effect of insulin in adipocytes are deficiency in the cGMP-inhibited low-Km cAMP phosphodiesterase, the insulin-activated serine protein kinase(s), which is thought to regulate this phosphodiesterase [Degerman et al., 1990; Smith et al., 1991], or in the serine/threonine protein phosphatases involved in the dephosphorylation of HSL.

There are several possible mechanisms for how HSL, the rate-limiting enzyme in fatty acid mobilization [Fredrikson et al., 1981; Langin et al., 1993; Albrink, 1974], could be involved in the development of lipodystrophy, including increased expression and enhanced activation upon phosphorylation of the activity-controlling site Ser-551 [Albrink, 1974] by cyclic AMP-dependent protein kinase. The suprapubic adipose tissue sample from the present patient exhibited HSL activity in the normal range for human adipose tissue [Frayn et al., 1993]. Provided that this adipose depot is representative of the atrophic adipose tissues, this argues against the possibility that HSL is overexpressed in the lipodystrophic tissue. In the same adipose tissue sample the activation of HSL upon phosphorylation by cyclic AMP-dependent protein kinase appeared to be somewhat increased. However, due to the fact that the increase was not statistically significant and that the control tissue was from another patient, it is difficult to assess whether this finding is of clinical importance. More patients, and preferably cases of partial lipodystrophy where lipodystrophic and control tissue can be obtained from the same patient, will have to be studied in order to determine whether the activation through phosphorylation is increased in lipodystrophic tissues in general.

Second, concerning PMDS, we should stress the heterogeneity of this disorder. When AMH cannot be detected either by immunoreactive or bioactive assays, a gene mutation affecting the synthesis or the stability of the AMH molecule is the most likely cause of the disorder [Imbeaud et al., 1994a]. Conversely, when normal AMH is produced by the testis, Müllerian duct insensitivity is considered the cause of PMDS [Josso et al., 1993]. The case we describe in the present work is a clear example of the latter, since AMH levels were normal and no mutations were found in the AMH gene. A possible cause for end-organ insensitivity in this subgroup of patients is the existence of abnormalities in the AMH receptor. Like the receptor of the other members of the TGFβ superfamily [Massagué, 1992], the AMH receptor, of which the gene has been recently cloned in the rabbit and in man, is a serine/threonine kinase with a single transmembrane domain [di Clemente, 1994; Imbeaud, 1994b] and will be screened in our patient in the near future.

Third, concerning HVDRR, the patient appears to be resistant to $1.25(OH)_2D_3$ action. However, when circulating $1.25(OH)_2D_3$ concentrations were increased to extremely elevated levels by pharmacological doses of exogenously administered Rocaltrol®, his hypocalcemia and rickets were reversed. The absence of hypercalcemia in the face of highly increased $1.25(OH)_2D_3$ is evidence for substantial resistance to $1.25(OH)_2D_3$. To investigate the nature of the patient's resistance, we examined his VDR and responsiveness to $1.25(OH)_2D_3$ in cultured fibroblasts. The VDR are normal in size and amount and show only a minor decrease in affinity for $1.25(OH)_2D_3$. In a test of responsiveness to hormones, the cells show a shift in the dose-response characteristics to the right requiring higher $1.25(OH)_2D_3$ to achieve a response. Induction of 24-hydroxylase mRNA is easily detectable at 1 nM in controls and requires 10 nM in the patient. Both binding and function experiments are consistent with a subtle defect in the VDR which results in a decreased affinity for $1.25(OH)_2D_3$. The decreased affinity and decreased responsiveness seem approximately 2- to 5-fold. This defect would most likely be a point mutation in the steroid binding domain of the VDR. Such an abnormality would predict that a rise in circulating $1.25(OH)_2D_3$ concentrations to 2- to 5-fold above normal would overcome the defect and achieve normal receptor occupancy and hormone responsiveness. Since the patient appears to be much more resistant to $1.25(OH)_2D_3$ and also concurrently has two other genetic diseases, it is likely that an additional abnormality is affecting his responsiveness to $1.25(OH)_2D_3$. The nature of this abnormality will require further study.

The concurrence of three defects of hormone responsiveness in a single patient is highly unusual. The explanation may be either the occurrence of two or three rare autosomal recessive mutations, or, more likely on a statistical basis, the presence of a single defect causing the three abnormalities. Since the known receptors involved in the three pathways are on different chromosomes (chromosome 12 for VDR and AMHR and chromosome 19 for insulin receptor), a major deletion or chromosomal rearrangement seems unlikely and could not be detected on chromosome analysis. In addition, the receptors for insulin and vitamin D appear relatively normal or mildly affected. Therefore, the best explanation would be a defect in a general hormone response mechanism common to vitamin D action, insulin action, fat deposition, and Müllerian regression. The putative defect would likely be distal to the receptor in the hormone action pathways. For example, such an abnormality would be analogous to a mutation in $G_s\alpha$ protein causing multiple abnormalities in Albright's hereditary osteodystrophy or McCune-Albright syndrome [Weinstein et al., 1991; Levine, 1991] or another regulatory protein [Brunetti et al., 1993]. Molecular studies are in progress to determine the nature of the defect underlying this unusual syndrome.

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